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Wnt genes encode a large family of secreted signaling molecules related to the *Drosophila* pattern regulating gene *wingless*. Several members of the *Wnt* family are implicated in mammary tumorigenesis suggesting that Wnt-activated pathways are a normal component of mammary gland development. Consistent with this hypothesis, we have demonstrated that seven *Wnt* genes are expressed in the developing mammary gland. One of these, *Wnt-7a*, appears to be required for epithelial branching in the adult mammary gland. In contrast, *Wnt-6*, *Wnt-10a and Wnt-10b*, are expressed at the earliest stages suggesting roles in induction and growth of the embryonic mammary gland. Recent evidence indicates that Frizzled members encode Wnt-receptors, and that Lef-1 and Lmx-1b are transcriptional mediators of Wnt-signaling. In this regard we have demonstrated that Fz-6 is expressed in the embryonic mammary gland, consistent with transducing Wnt signals, and *Lmx-1b*, like *Lef-1*, is required for mammary gland development. We are using a combination of transplantation and transgenic procedures to further explore these pathways. Our hope is that a better understanding of normal development of the mammary gland will provide new insights into possible mechanisms underlying tumor formation.

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5. INTRODUCTION

Nature of problem/Background of previous work.

The mammary gland develops from an epithelial outpocketing of the ventral ectoderm at 11dpc in the mouse embryo [1-4], in response to an initial inductive signal from the underlying mesenchyme [5]. In the female mouse embryo, there is little change in the primary bud over the next four days. However, in the male, mesenchyme surrounding the epithelium condenses from day 14 and this is followed by a rapid necrotic degeneration of the epithelial rudiment. Tissue recombination experiments have convincingly demonstrated that this process is dependent upon testosterone [6], and correlates with the acquisition of testosterone receptors by the mammary mesenchyme [7], which occurs in response to epithelial derived signals [8], and the initial production of testosterone by the embryonic testes. In addition to testosterone responsiveness, the epithelium also induces estrogen responsiveness but, at this stage of development, there appears to be no in vivo role for estrogen [1]. Thus, by the end of this resting period, which is characterized by the appearance of the mammary bud (16.0 dpc), the female mammary gland is poised for further development whereas the male gland is destroyed.

From 16.0dpc to 2dpp, the mammary epithelium extends as the primary mammary sprout into the mesenchyme reaching the fat pad precursor where epithelial branching is initiated. The trigger for elongation of the mammary epithelium is not known, however outgrowth follows the resumption of proliferative activity in the epithelium. The trigger for epithelial branching clearly resides in the mesenchyme of the fat pad precursor. Epithelial morphogenesis, which forms slim epithelial ducts with secondary lateral buds, is specific to the fat pad mesenchyme [9]. Growth into other sources of mesenchymal tissue *in vitro* , e.g. salivary mesenchyme, produces epithelial outgrowths typical of the organ from which the donor mesenchyme was removed. [10]. Thus, shortly after birth in the mouse, and in other mammals [2], the female mammary gland consists of a primary duct connecting with a rudimentary branched epithelium within the presumptive fat pad.

From the period after birth to approximately 4 weeks pp there is very limited growth of the branching epithelium of the mouse mammary gland. However, at 4 to 6 weeks, coupled with the acquisition of sexual maturity, there is a period of extensive cell growth in which the epithelial ducts elongate and branch, extending throughout the fat pad. [11,12] Renewed growth correlates with the reappearance of end buds, a monolayer of unspecialized epithelium at the ends of the ducts. The end buds are thought to contain stem cells which generate differentiated ductal epithelium and myoepithelial cells of the gland. Thus, post natal branching morphogenesis is regulated largely at the termini of the ducts, by controlling the proliferative activity of the end buds. End bud activity is in turn dependent upon ovarian hormones, as ovariectomy results in a rapid loss of end buds and cessation of growth [11,12].

After reaching sexual maturity (6 to 8 weeks pp), further ductal development stops until pregnancy is established. At this time, a second extensive period of ductal growth and branching occurs to fill all the remaining interductal space in the fat pad. During the later phase of pregnancy there is an accompanying development of lobuloalveolar epithelium. Along with the morphogenetic changes in the gland during pregnancy, there is a progressive development of secretory epithelium, such that by birth, a fully functional lactogenic epithelium is established. Interestingly, cytodifferentiation of secretory epithelium will occur in vitro in the absence of morphogenesis, indicating that the two processes are not mutually dependent [13]. Finally, on cessation of suckling, there is a massive involution of the mammary gland due to a widespread destruction of epithelial tissue and the cycle of branching morphogenesis is repeated at the next round of pregnancy.

The mammary gland is unusual, with respect to most organs, in that most of its growth occurs in the adult, and that there are cyclical periods of growth and regression. The control of these processes has been extensively studied and compelling evidence exists for complex regulation mediated by systemic hormonal signals, and locally acting peptide growth factors (for review see [11,12]).

The initial observation that ovariectomy leads to a cessation of end bud growth implicated hormones in the control of mammary development. There is an absolute requirement for estrogen for proper development of epithelial branching. Maximal growth also appears to require growth hormone or prolactin [11,12]. However, whether these hormones act directly, or sensitize the epithelium to the action of other factors, is not clear. Lobuloalveolar growth requires, in addition to the above, progesterone which accumulates later in pregnancy. Finally, the onset of lactation correlates with the increase in prolactin and glucocorticoids and a decrease in progesterone [11,12].

Evidence for involvement of peptide growth factors in the regulation of mammary development has come from the direct observation of growth factor expression, and implant and transgenic studies which have manipulated growth factors in the mammary gland. Slow release implants of EGF stimulates local growth of end buds in quiescent mammary epithelium [14], whereas implantation into growing mammary glands causes local inhibition of ductal growth, and a down regulation of EGF receptors [15]. Thus, EGF may have a dual specificity depending upon the particular stage of development. TGF-β1 implants also suppress ductal growth [16] acting specifically on the end buds to inhibit DNA synthesis [17] whereas TGFα stimulates alveolar and ductal growth [18-21].

Additional evidence for peptide factors in growth regulation has come from the analysis of mammary tumors in which growth controls have been uncoupled following expression of genes not normally active in the mammary gland. A number of loci, have been shown to undergo MMTV mediated insertional activation in mouse mammary tumors (for review see [22]). Four of these encode secreted peptide factors, *Wnt-1* [23] and *Wnt-3* [24] members of the *Wnt*-gene family, and *FGF-3* [25] and *FGF-4* [26], members of the fibroblast growth factor family. Additional evidence suggests that *Wnt* and *FGF* genes may cooperate in tumor formation as frequently *Wnt-1* and *FGF-3* are co-activated in the same mammary carcinomas [27].

The oncogenic role of *Wnt-1* has been demonstrated by *in vitro* and *in vivo* studies. Transfection of the *Wnt-1* gene into C57MG cells, a primary mammary epithelial cell line, leads to morphological transformation [28,29]. However, these cells do not grow in soft agar or form tumors in syngeneic hosts. In contrast RAC311C cells are rendered morphologically transformed and tumorigenic when transfected with *Wnt-1* [30]. Formal proof of the transforming roles of Wnt-1 has come from transgenic studies which lead initially to hyperplasia, in both the male and female mammary gland, and progress to the formation of adenocarcinomas [31]. As was observed in spontaneously occurring tumors, there is also a synergistic affect of *FGF-3* on *Wnt-1* transformation in the transgenic model [32].

In addition to *Wnt-1* and *Wnt-3*, fourteen additional members of the mouse *Wnt*-gene family have been identified. Human Wnt-2 was isolated serendipitously in a search for the cystic fibrosis gene [33,34]. Like *Wnt-1* and *Wnt-3*, Wnt-2 is implicated in tumorigenesis as it appears to be amplified and highly expressed in some MMTV induced tumors [35]. Amplification appears not to be related to MMTV, but is a novel mechanism which presumably acts in conjunction with MMTV activated genes to transform epithelial cells [35]. *Wnt-3a* was identified on the basis of its close relationship to *Wnt-3* [36], and *Wnt's-4, 5a, 5b, 6, 7a, 7b*, on the basis of a PCR cloning approach [37] which has been successful in identifying *Wnt*-genes in many species, as well as two new mouse members (*Wnt-10* and *11*; A. McMahon, unpublished data).

All Wnt-proteins have several features in common including a putative signal peptide sequence, one conserved glycosylation site, and 20 absolutely conserved cysteine residues. Typically Wnt proteins are 38 to 45kd. Although only *Wnt-1* and *Wnt-2* have been studied, and these analyses have been restricted to cell culture systems, both genes appear to encode poorly secreted glycoproteins with strong affinity for cell surface and/or extracellular matrix [29,38-44]. Thus, it is likely that they are involved in short-range signaling. Functional analyses of several members indicates these important regulatory roles in invertebrate and vertebrate development [reviewed in 45,46].

The observation that *Wnt* expression leads to morphological transformation of mammary epithelial cells *in vitro* and hyperplastic growth *in vivo* indicates that mammary epithelium is responsive to *Wnt* gene products. If, Wnt-proteins act as signals (a conclusion greatly strengthened by studies on the Drosophila Wnt-1 orthologue *wingless*, [46]), then by analogy with other families of peptide signals, it would seem likely that the responsiveness of mammary epithelium reflects the expression of functional Wnt-receptors.

Recent evidence demonstrates that unlike *Wnt-1* and *Wnt-3*, six family members are expressed, and developmentally regulated, during normal adult mammary gland development [47]. Thus, the responsiveness to ectopic expression of *Wnt-1* or *Wnt-3* presumably reflects some modulation of *Wnt*-signaling pathways which normally respond to endogenously expressed Wnt-factors. For example, if Wnts normally stimulate cell growth, ectopic expression of *Wnt-1* or *Wnt-3* may lead to hyperstimulation of a proliferative Wnt-signaling pathway. Conversely, if endogenously expressed *Wnts* suppress proliferative activity, ectopic *Wnt-1* or *Wnt-3* expression may block Wnt-mediated growth suppression, possibly by interfering with receptor function.

The situation is likely to be complex on the basis of our studies of *Wnt*-transcription in the adult mammary gland [47b] . *Wnt-2* expression is very weak and confined to virgin or nonpregnant mice [47b]. Thus although *Wnt-2* causes C57MG cell transformation, its expression does not correlate with proliferative activity. Quite the opposite, it is limited to the quiescent state. *Wnt-5a* and *Wnt-7b* are also expressed at low levels in virgin mice [47]. However, expression extends into mid but not late pregnancy showing decreasing levels of expression despite the large increase in mammary epithelium. In contrast, *Wnt-5b* and *Wnt-6* are expressed at low levels prior to pregnancy and increase considerably to midpregnancy, declining by parturition [47]. Thus, these two members show a better correlation with epithelial expansion. Finally *Wnt-4* expression is uniform from in the virgin gland until late in pregnancy when it rapidly declines [47].

Transformation assays on C57MG cells indicate that several Wnt-members which are normally expressed in the mammary gland are transforming in this assay [48]. Wnt-2, -5b and -7b are moderately transforming, weaker than Wnt-1, 3a and 7a, whereas Wnt-4, 5a and 6 are non transforming. *Wnt-4* and *Wnt-5a* are normally expressed by C57MG cells, thus elevation of endogenous expression several fold does not lead to transformation. These results suggest that hyperplasia *in vivo* may result from inappropriate activation of Wnt-2, Wnt-5b and/or Wnt-7b signaling pathways.

In summary, the data clearly support a model in which normal mammary, epithelial growth is regulated by one or more *Wnt*-genes. They demonstrate that uncoupling of these regulatory pathways leads to hyperplasia [31,49] and adenocarcinomas *in vivo* [31]. However, without a better understanding of the normal spatial expression of Wnt-proteins and their putative receptors, and the transforming activity of the family as a whole *in vivo*, we are not in a position to grasp the full significance of their functions in the normal and transformed mammary tissue, nor the relevance that this family may have to human breast cancer.

Purpose of present work/Methods of approach

As discussed above it is now over ten years since Nusse and Varmus identified a locus in the mouse associated with the generation of mammary tumors. It is now clear that the associated gene, *Wnt-1*, is one member of a large family of putative signaling molecules which normally regulate embryonic development. Several members have now been implicated in epithelial cell transformation in the mammary gland from the analysis of spontaneously occurring mouse tumors (*Wnt-1, Wnt-3, Wnt-3a*), transgenic experiments (*Wnt-1*) and *in vitro* studies (*Wnt-1, 2, 3, 3a, 5b, 7a, 7b*). Thus, it would appear that hyperplasia, and eventual adenocarcinoma formation, in the mouse mammary gland result when normal growth regulatory pathways which are presumably controlled by Wnt-proteins, are perturbed by deregulated expression of certain Wnt-family members.

Understanding growth control in the mammary gland is essential for designing strategies which will treat mammary tumors. Further, potential growth regulators are likely mediators of mammary transformation, as exemplified by studies on *Wnt*-genes in the mouse, and should thus be examined for contributory roles in human mammary cancer. This proposal set out to examine the normal and oncogenic roles of Wnt protein in the mammary gland of the mouse and human, and to dissect the Wnt-regulatory pathways at the receptor level. Specifically we proposed to address the issue of whether *Wnt*-genes may be involved in human cancers by directly examining expression in mammary tumors using Northern blot analysis. We propose to use transgenic mice to examine the relationship between normal *Wnt*-gene expression and mammary transformation. As Wnt-signaling is most likely a conventional receptor-mediated process, ectopic expression of specific Wnt-signals presumably exerts its effects through one or more receptor pathways coupled to endogenously expressed Wnt-proteins. If so, we should be able to identify a likely candidate pathway by assaying the transforming potential of endogenously expressed Wnt-proteins when their normal regulation is uncoupled, either by ecoptic expression or gene ablation. Moreover, characterizing the normal expression of *Wnt*-genes and their products in relation to the developing mammary gland may provide strong suggestive evidence as to what growth regulatory pathways may be responsive to Wnt-signals. Finally we propose several approaches toward identifying Wnt-receptors which will be an essential step in fully defining Wnt-signaling pathways, and their regulatory function in the mammary gland. Thus, the proposed studies are directly relevant to the issue of the genetic alterations involved in the origin and progression of cancer and the changes in cellular and molecular function which may account for the development and progression of breast cancer.

In summary we proposed five specific goals

1) To determine, using transgenic mice, which if any of the *Wnt*-members normally expressed in the mammary gland are oncogenic when ectopically expressed using an MMTV enhancer construct.

2) To determine the relevance, if any, of *Wnt-5b* in normal gland development by studying mice homozygous for a likely null mutation in the *Wnt-5b* gene.

3) To determine the normal temporal and spatial expression of *Wnt* genes, and their protein products, during embryonic and adult mammary gland development.

4) To use various schemes to attempt to identify other proteins, particularly candidate receptors, which interact with Wnt-proteins.

5) To isolate sequences encoding all of the yet-unidentified human *Wnt*-genes, providing clinicians with a broad array of Wnt-probes which may be important in the analysis of human mammary carcinomas.

6. BODY

Over the past three years we have made substantial changes as a result of ours and others findings. This lead to a change in our goals as reported in the previous reporting period. we have dropped some of the original goals.

1) Transgenic analysis of Wnt-mediated oncogenesis

As we discussed in last years report, this work was discontinued in favor of a study of *Wnt-6* and the newly identified *Wnt-10* in embryonic mammary gland development (see later).

2) *Wnt-5b* mutant analysis

As discussed in the last funding period, we generated Wnt-5b mutants but found that females showed no discernible phenotype. We recognize that there is a slight chance that the allele is not a null allele, and this possibility is being followed up in collaboration with me former postdoctoral fellow, Dr. Shinji Takada.

3) Wnt expression in the mammary gland

Although we have demonstrated that several *Wnt* genes are expressed during mammary development, we do not know in which cell types, nor the spatial details. This is of critical importance. Growth and branching morphogenesis is primarily regulated at the end buds. Thus, any growth stimulatory or growth repressive action of a Wnt member is likely to act on this aspect of the epithelial network. As the available evidence suggests that Wnts are short range factors, we would therefore anticipate that some Wnt members will be locally distributed either in the stroma surrounding the end buds, or perhaps in the end bud themselves, and their expression would be predicted to change dramatically with development. As reported in the previous period, we have attempted, unsuccessfully to examine their expression with antibodies. In this period we have decided to approach their potential roles genetically. As discussed earlier, we had previously demonstrated that Wnts-4, 5a, 5b, 6, and 7b are expressed in the adult mammary gland. Although not detected in the mammary gland in the original study, Wnt-7a is expressed in mammary epithelial cell lines. We have mutants in all but Wnt-6. Over the past year we have had several unsuccessful attempts to target Wnt-6. It is not clear where the problem lies as the targeting constructs have used isogenic DNA and large areas of flanking homology, but we have screened over 400 colonies with no homologous recombinants detected. This work will continue, most likely with modified vectors using additional flanking regions.

Of the other Wnts, mutations in Wnt-4, Wnt-5a and Wnt-7b are recessive lethals. Wnt-7b at 9.5 dpc, the other two at birth. Clearly this presents an obstacle to examining their roles in the adult, one which we are attempting to overcome in collaboration with Dr. Cathrin Briskin in Dr. Robert Weinberg's laboratory at the Whitehead Institute. Dr. Briskin is transplanting the mammary epithelium at birth into cleared fat pads of wild type weanlings. Initial results with Wnt-4 indicate that Wnt-4 is not required for mammary development, though to be absolutely certain we are repeating these experiments using genetic markers (lacZ) to distinguish between graft and host tissue. We will also extend this approach to Wnt-5a, and possibly to Wnt-7b mutants which we have recently been able to rescue to birth by tetraploid aggregation.

The most interesting results relate to the viable mutant Wnt-7a. An examination of the mammary glands in females at 6 weeks of age has revealed a striking reduction in tertiary branching (see Fig 1 in appendix). We are following up on this observation in collaboration with Dr. Briskin by transplanting the mammary epithelium from these mice into the cleared fat pads of wild type females to examine the requirement for Wnt-7a during pregnancy and lactation. This is necessary as the Wnt-7a homozygous females have a defect in uterine development that precludes implantation.

We have been studying Wnt-7a for the past two years in relation to its role in limb patterning. Here Wnt-7a is a dorsalizing signal supplied by the dorsal ectoderm operating on a downstream

mesenchymal target, a transcriptional regulator, Lmx-1b. We have examined Lmx-1b in the mammary gland, and discovered that it is present from the initiation of mammary development at 11.5 dpc (Fig. 2). In conjunction with our colleague Dr. Randy Johnson at M. D. Anderson, in Texas, we have looked at mammary gland in Lmx-1b mutants generated in his group. Interestingly, they lack any detectable mammary gland at 18.5 dpc (Fig. 3). We will determine whether this reflects a complete failure of mammary gland induction, or a secondary failure post-initiation. Clearly, the phenotype is more severe than that seen in Wnt-7a mutants. However, one possible explanation is that Lmx-1b may have distinct Wnt-signals regulating expression at different stages of development. In addition to addressing Lmx-1b expression in the adult mammary gland of wild-type females and Wnt-7a mutants, we have explored the expression of Wnts at the onset of mammary gland development when Lmx-1b is first required. As reported in the previous period these studies identified Wnt-*6* and *Wnt-10*.

Expression of *Wnt-6* is not restricted to the mammary bud epithelium but is present in all ectoderm, suggesting that it may play a widespread role in epithelial signaling. In contrast *Wnt-10* is expressed specifically at two sites of mesenchymal-epithelial interactions, the tooth and mammary buds (Figure 2 in Appendix). Thus, *Wnt-10*, which is a novel mouse family member identified in our PCR-based screens, is a prime candidate for a *Wnt* which might actually induce mammary gland development.

We proposed in the last funding period to generate mutants in Wnt-10. We successfully targeted Wnt-10 in ES cells, but the single clone did not go through the germ-line. We also became aware of results from Dr. Phil Leder's and Dr. Greg Shackleford's laboratories. They had identified an additional Wnt-member, very closely related to Wnt-10. We now refer to our original clone as Wnt-10b and the closely related gene as Wnt-10a. The Leder laboratory has mutated Wnt-10b. Females are viable and nurse their offspring. Thus, clearly Wnt-10b is not essential for mammary gland development. We have examined the expression of Wnt-10a (Fig.2). Clearly, these two Wnts are co-expressed in the developing mammary gland. Thus, they may well be functionally redundant. We have spoken with Dr. Leder and agreed on a collaboration that if we generate Wnt-10a mutants, we will then intercross these with his Wnt-10b mutants, assuming no mammary phenotype in the single mutant.

To explore the possibility that Wnt-10 signaling may regulate Lmx-1b induction in the mammary gland we are adopting two approaches. In the first we have generated a transgenic construct which places Wnt-10b under the regulation of the keratin-14 promoter. This will result in the ectopic expression of Wnt-10b throughout the epidermis, most notably along the entire length of the mammary ridge. We will then determine whether this results in ectopic activation of Lmx-1b, and ectopic mammary gland development. The model follows the precedent of ectopic limb formation along the flank in response to FGF. In the second approach we will graft foci of Wnt-10b expressing cells into the mammary ridge and culture these regions in an explant system we have developed.

Finally with respect to general genetic tools that will allow genetic manipulation in the mammary epithelium we propose to generate several new strains. In the first of these we will use the K14 promoter to express the yeast transcriptional activator GAL4 and P1 cre recombinase in the epithelium to allow ectopic activation and knockout of gene function in the early mammary epithelium. These constructs have been made and the lines are being generated. Further, if time and resources permit, to gain greater mammary specificity, two "knock-in" lines will be constructed in which GAL4 and cre are placed under the control of the Wnt-10b regulatory regions. [50].Together these approaches will allow the investigator to analyze loss-of-function mutants in genes suspected of a role in mammary epithelial development, but which have an essential requirement at some earlier stage of development. One particular use of these lines is likely to be in exploring Wnt receptor action (see below).

4) **Wnt receptors**

The last year has seen the identification of members of the frizzled family as likely Wingless/Wnt receptors [51]. As a consequence of this observation we examined mammalian Frizzled expression and found that of the identified Frizzleds, only Frizzled-6 is expressed in a mammary gland specific pattern in the early mammary gland. Thus, Frizzled-6 is a candidate for a Wnt-6 or

Wnt-10 receptor. We envisage a number of experiments to investigate the relationship of Frizzled-6 to Wnt-signaling in the mammary gland. In conjunction with Wnt-10 and Wnt-6 we will determine the normal distribution of Frizzled-6 at different periods in mammary gland development. We will attempt to determine whether either of these Wnts can interact with Frizzled-6, and whether such an interaction is specific to this Frizzled. Interestingly, Frizzled-6 does not appear to bind to wingless, in contrast to other mammalian Frizzleds [51]. Thus, one approach would be to determine whether Wnt-6/10 and Frizzled co-injection into Xenopus embryos can elicit phenotypes that either component alone is incapable of generating, Using this approach Dawid and colleagues have demonstrated that co-injection of Frizzled-5 and Wnt-5 into the Xenopus embryo results in axial duplication, a response normally elicited by the Wnt-1 class of signals, but only elicited by Wnt-5a in conjunction with Frizzled-6. Finally, using the genetically engineered strains of mice discussed above, we may be able to perform functional experiments to address the role of Frizzled-6 in regulating the mammary gland. For example, ectopic expression of Frizzled-2 in Drosophila is sufficient to activate the wingless pathway. Does ectopic activation of Frizzled-6 lead to mammary hyperplasia? Further, Frizzled binding to Wnts maps to a cysteine-rich extracellular domain [51]. Does expression of this domain in the mammary gland act as a dominant negative arresting growth of the gland?

5) **Human *Wnt*-clones**

As discussed in the last funding period, our reagents have been distributed to other groups better able to study the human side of Wnts in mammary gland while we focus on the mouse.

7. CONCLUSIONS

There are several implications of the studies thus far.

1) **Transgenic analysis of Wnt-mediated oncogenesis**
In the light of our discoveries and those of other groups in the last year, we believe that our resources are best focused on other aspects of the proposal which are likely to lead to more original findings. It is important to know whether Wnt-10a or Wnt-10b is capable of transforming mammary epithelium. However, the experiment has apparently been performed by nature as a recent report indicates that MMTV activation of Wnt-10b (as originally shown for Wnt-1), leads to mammary tumorigenesis [53].

2) ***Wnt-5b*** **mutant analysis**
There is no obvious phenotype in mice homozygous for an insertion in the *Wnt-5b* locus. It remains possible that this is not a null allele. Alternatively, Wnt-5b may not be essential for mammary gland development. Our studies have identified Wnt-7a as a possible mediator of epithelial branching-growth in the adult mammary gland and three Wnt-members, Wnt-6 and Wnt-10a and Wnt-10b are excellent candidates for regulating the initial development of the mammary gland. We will explore the roles of Wnts by mammary transplantation, transgenic and gene targeting approaches to address both their functions and the pathways by which they act. Further, we will generate strains of mice which will allow mammary epithelial gene expression to be modified from its earliest stages. These reagents should be of great value in the community and will be used in this proposal to look at aspects of Frizzled action. The relevant DNA constructs will be generated for introduction into mouse ES cells.

3) ***Wnt*** **expression in the mammary gland**
We will attempt to complete the picture of Wnt/Fz/Lmx-1b expression in the developing mammary gland, using wholemount in situ hybridization. This will be essential to understanding the possible regulatory relationships that may exist in development of the organ.

4) **Wnt receptors**
The problem of how Wnt-proteins signal, most specifically their receptors, is important not only to our understanding of the many important roles that Wnt-proteins play in the regulation of invertebrate and vertebrate development, but also to their oncogenic actions in the mammary gland. The identification of Frizzles as candidate Wnt-receptors, and more specifically our identification of Frizzled-6 as a mammary specific Frizzled, paves the way for examining the role of Frizzles in mammary development. To this end we will complete our general survey of Frizzled expression and a more detailed analysis of the expression of Frizzled-6 in conjunction with Wnts. In addition, we will determine whether Frizzled -6 interacts with Wnt-6/10, and if so, whether this interaction is specific.

5) **Human** ***Wnt*****-clones**
We have not pursued the cloning of additional human *Wnts* as this would duplicate efforts in other groups that we have become aware of since submission of this grant. However, we will continue to share unpublished data to facilitate a rapid follow up of potentially interesting areas such as the expression of *Wnt-10*a and b which may have relevance to human breast cancer, in our collaboration with groups in England.

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9. Appendix

Figure 1. Low power (left) and high power (right) panel views of epithelial branching in wild-type (upper) and Wnt-7a mutant (lower) mammary glands at 6 weeks post partum. Note the absence of tertiart branching in the mutant epithelium.

Figure 2. Intiation of mammary gland development at 12.5 dpc is associated with expression of Wnt-6, -10a, and -10b, Fz-6 and two potential transcriptional targets, Lmx-1b and Lef-1.

Figure 3. By 18.5 dpc the mammary gland of wild-type females has involuted (left) and branched several times in the underlying mesenchyme of the fat pad (center). In the absence of Lmx-1b, no mammary gland development is observed in the region of the fat pad (right).

Figure 1

DAMD 94-J-4453

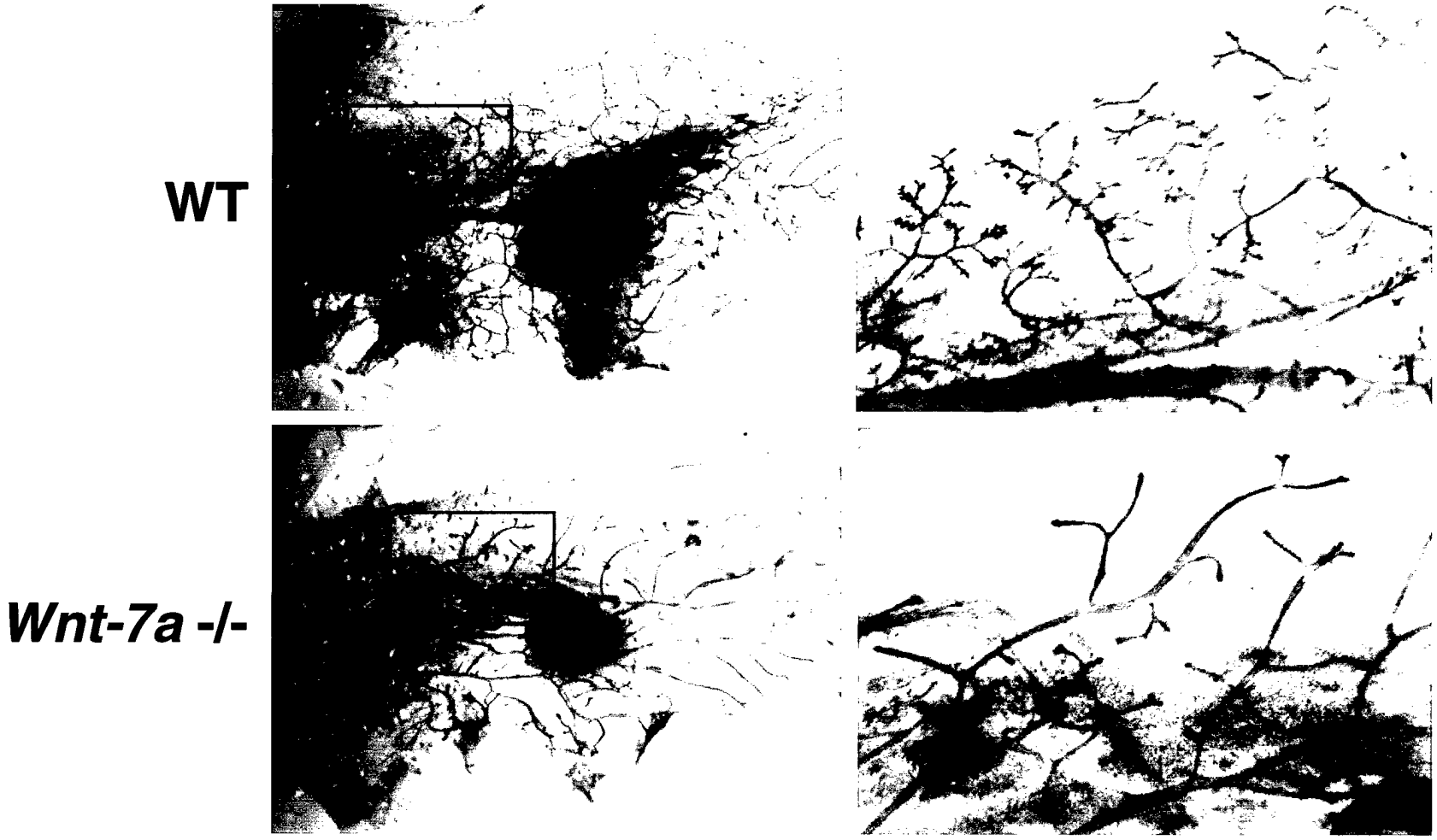
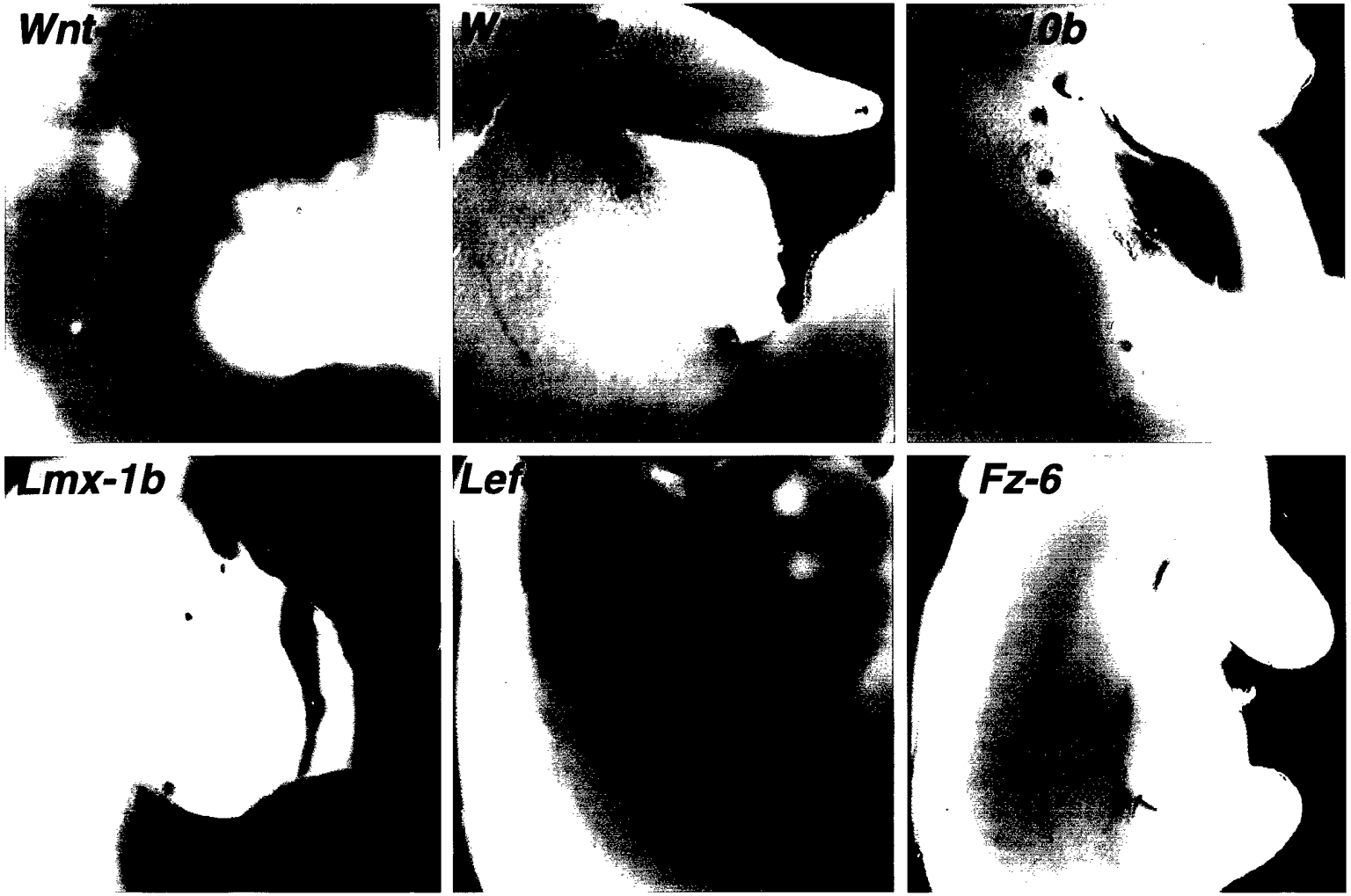


Figure 2

DAMD 94-J-4453



Lmx1b Regulates Mammary Gland Development

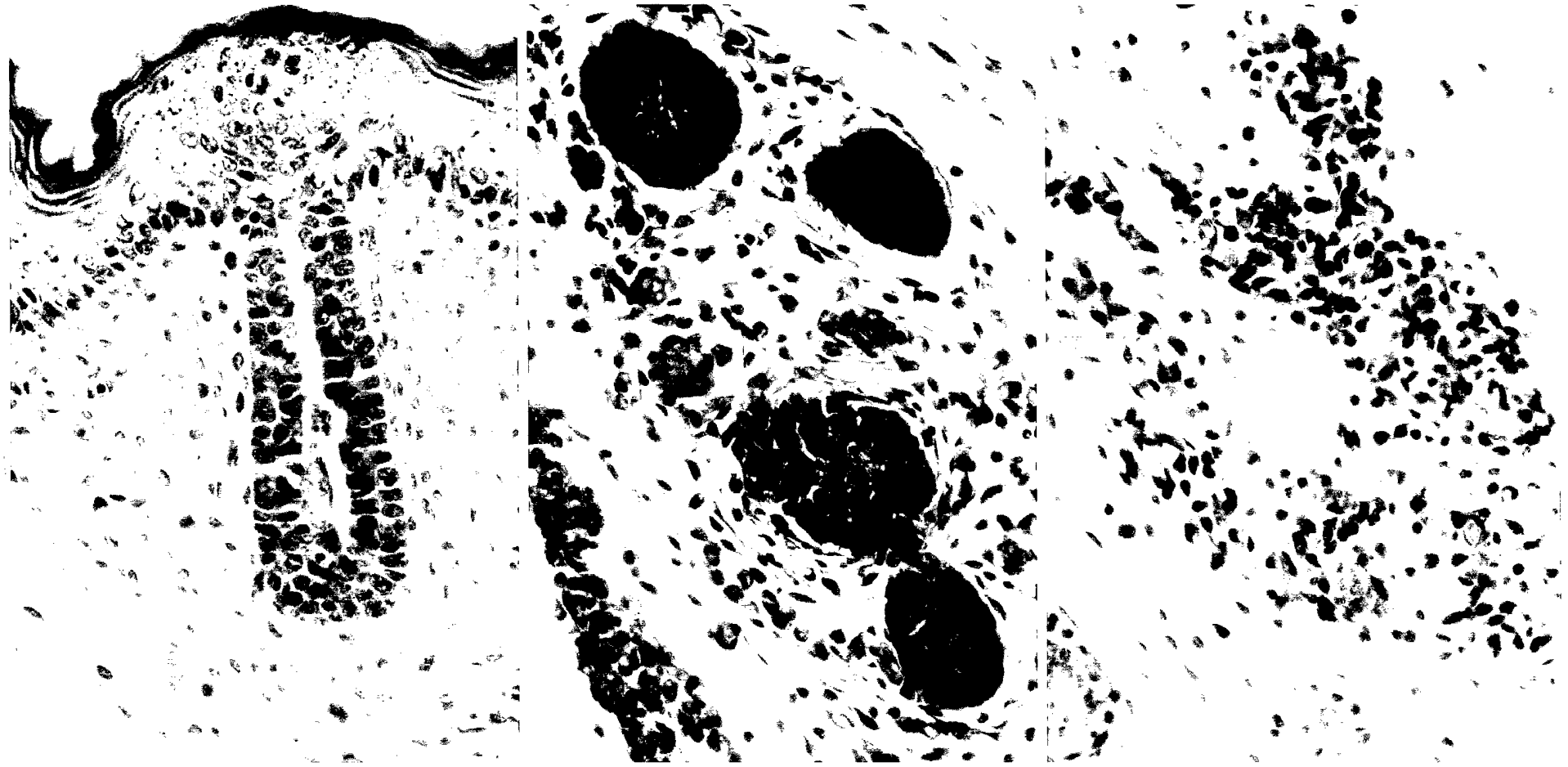


Figure 3

DAMD 94-J-4453